United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol  

SAM 116  

Supplemental Assay Method for the Phenotypic Examination of Pseudorabies Virus for Thymidine Kinase Activity by a Plaque Selection Method  

Date: October 16, 2014  
Number: SAM 116.05  
Supersedes: SAM 116.04, February 9, 2011  
Standard Requirement: 9 CFR 113.318  
Contact: Alethea M. Fry, (515) 337-7200  
Peg A. Patterson  

Approvals:  
/s/Geetha B. Srinivas ___________________________ Date: 08Dec14  
Geetha B. Srinivas, Section Leader  
Virology  
/s/Byron E. Rippke ___________________________ Date: 16Dec14  
Byron E. Rippke, Director  
Policy, Evaluation, and Licensing  
Center for Veterinary Biologics  
/s/Rebecca L.W. Hyde ___________________________ Date: 16Dec14  
Rebecca L.W. Hyde, Section Leader  
Quality Management  
Center for Veterinary Biologics  

United States Department of Agriculture  
Animal and Plant Health Inspection Service  
P. O. Box 844  
Ames, IA  50010  

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1. Introduction

This Supplemental Assay Method (SAM) describes an in vitro assay method which uses a selective media in a cell culture system to detect the presence or absence of extraneous thymidine kinase (TK)-positive pseudorabies virus (PRV) in a thymidine kinase-negative (TK-), modified-live PRV vaccine.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Water bath

2.1.2 Incubator, 36°C ± 2°C, high humidity, 5 ± 1% CO₂

2.1.3 Microscope, inverted light

2.1.4 Microscope, ultraviolet (UV) light

2.1.5 Freezer, ultra-low

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Connective tissue, mouse, TK mutant (L-M[TK-]) cells

2.2.2 Madin-Darby bovine kidney (MDBK) cells or other susceptible cells

2.2.3 PRV Reference, Shope strain

2.2.4 Minimum essential medium (MEM) (National Centers for Animal Health (NCAH) Media #20030)

1. 9.61 g MEM with Earles salts without bicarbonate

2. 1.1 g sodium bicarbonate (NaHCO₃)
3. Dissolve with 900 mL deionized water (DI).

4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to 60°± 2°C until dissolved. Add to Step 3 with constant mixing.

5. Q.S. to 1000 mL with DI; adjust pH to 6.8-6.9 with 1N hydrochloric acid (HCl).

6. Sterilize through a 0.22-µm filter.

7. Aseptically add:
   a. 50 µg/mL gentamicin sulfate
   b. 10 mL L-glutamine (200 mM)

8. Store at 2°-7°C.

2.2.5 Growth Medium

1. 900 mL of MEM

2. Aseptically add 100 mL gamma-irradiated fetal bovine serum (FBS)

3. Store at 2°-7°C.

2.2.6 Hypoxanthine, aminopterin, thymine (HAT) Medium

1. 200 mL Growth Medium

2. 2 mL HAT media supplement (50X)

2.2.7 0.01 M Phosphate buffered saline (PBS) (NCAH Media #30054)

1. 1.19 g sodium phosphate, dibasic, anhydrous (Na₂HPO₄)

2. 0.22 g sodium phosphate, monobasic, monohydrate (NaH₂PO₄•H₂O)

3. 8.5 g sodium chloride (NaCl)

4. Q.S. to 1000 mL with DI.

5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 1 N HCl.

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6. Sterilize by autoclaving at 15 psi, 121°± 2°C for 35 ± 5 minutes.

7. Store at 2°- 7°C.

2.2.8 80% acetone

1. 80 mL acetone
2. 20 mL DI
3. Store at room temperature.

2.2.9 Tissue culture flask, 25-cm²

2.2.10 Pipette, 10-mL

2.2.11 Graduated cylinder, 25-mL, 50-mL, 100-mL, and 250-mL, sterile

2.2.12 Swine anti-PRV fluorescein isothiocyanate- labeled conjugate (PRV Conjugate)

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in aseptic techniques and cell culture growth and maintenance.

3.2 Preparation of equipment/instrumentation

On the day of testing, set a water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 A day prior to test initiation and a day prior to each of the 3 additional L-M(TK-) passages, seed 25-cm² flasks with L-M(TK-) cells, in Growth Medium, at a cell count that will produce a monolayer after 1 day of incubation. Cells used for seeding should be from monolayers that had been previously passaged every 3 to 4 days. Three L-M(TK-) control flasks and 1 L-M(TK-) flask are required for each Test Vaccine. Incubate at 36°± 2°C in a CO₂ incubator.
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3.3.2 A day before the last passage, seed 25-cm² flasks with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 1 day of incubation. Three MDBK control flasks and 1 MDBK flask are required for each Test Vaccine. Incubate at 36°± 2°C in a CO₂ incubator.

3.3.3 PRV Positive Control. On the day of test initiation, rapidly thaw a vial of PRV Reference in a 36°± 2°C water bath. Dilute the PRV Reference in MEM to contain 10⁴ 50% tissue culture infective dose (TCID₅₀)/100 µL.

3.3.4 Working PRV Conjugate. On the day of the fluorescent antibody (FA) Confirmatory Test, dilute the PRV Conjugate in PBS according to the Center for Veterinary Biologics (CVB) supplied Reagent Data Sheet.

3.4 Preparation of the Test Vaccine

On the day of test initiation, using a graduated cylinder, rehydrate a vial of the Test Vaccine with the supplied diluent. Incubate for 15 ± 5 minutes at room temperature.

4. Performance of the Assay

4.1 First passage on L-M(TK-) cells

4.1.1 On the day of test initiation, decant Growth Media from all the L-M(TK-) flasks except 1, which remains unopened as a cell control. Label the unopened flask “Cell Control.”

4.1.2 The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). Inoculate 1.0 mL of the Test Vaccine into an L-M(TK-) flask labeled with the Test Vaccine identification.

4.1.3 One mL of PRV Positive Control is inoculated into an L-M(TK-) flask labeled “PRV Positive Control.”

4.1.4 One mL of HAT Medium is inoculated into an L-M(TK-) flask labeled “Medium Control.”

4.1.5 Allow inocula to absorb by incubating flasks at 36°± 2°C for 60 ± 10 minutes in a CO₂ incubator.

4.1.6 After incubation, add 9.0 mL of HAT Medium to all flasks except the Cell Control (remains unopened). Incubate all flasks at 36°± 2°C for 4 days in a CO₂ incubator.
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4.1.7 During incubation, periodically observe all flasks for bacterial or fungal contamination. It is not necessary for microscopic observation of the L-M(TK-) cells. However, on examination, the L-M(TK-) cells will be rounded due to the HAT Medium. The Cell Control flask should remain normal.

4.1.8 After incubation, all flasks are frozen at -70°C ± 5°C for at least 2 hours. Flasks may be held at -70°C ± 5°C until the next passage. The Cell Control flask is discarded, as a new Cell Control flask will be used with each passage.

4.1.9 Thaw all the remaining flasks, with frequent shaking, at room temperature until completely thawed.

4.2 Repeat Steps 1 through 9 for a total of 4 passages, inoculating each flask with 1.0 ml of the thawed cell and media suspension of the appropriate control or Test Vaccine from the previous passage instead of the initial inocula.

4.3 Passage on MDBK cells

4.3.1 From the last thawed passage (4th passage) on L-M(TK-) cells, repeat Sections 4.1.1 through 4.1.3, except substitute MDBK cells for the L-M(TK-) cells and inoculate each flask with 1.0 mL of the 4th passage cell and media suspension of the appropriate control or Test Vaccine instead of the initial inocula.

4.3.2 Allow inocula to absorb by incubating at 36°C ± 2°C for 60 ± 10 minutes in a CO₂ incubator.

Note: Do not use HAT Medium.

4.3.3 After incubation, add 9.0 mL of Growth Medium to all flasks except the Cell Control (remains unopened). Incubate at 36°C ± 2°C for 4 days in a CO₂ incubator.

4.3.4 Observe all flasks daily with the inverted light microscope for typical PRV CPE. The CPE of PRV is visible as grape-like clusters of rounded cells in the cell monolayer. The PRV Positive Control flask should show typical PRV CPE after 4 days. Any Test Vaccine observed to have typical PRV CPE is considered a Suspect Test Vaccine.

4.4 Confirmation of PRV CPE in Suspect MDBK Test Vaccine flasks (FA Confirmatory Test). Conduct an FA Confirmatory Test using specific PRV conjugate on any Suspect Test Vaccine. The PRV Positive Control flask, the HAT Medium flask, and the Cell Control flask are similarly examined and used as controls. If CPE is not observed in a Test Vaccine, this procedure is not required.
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4.4.1 Decant Growth Medium from the MDBK flasks for the Suspect Test Vaccine(s), the PRV Positive Control, the HAT Medium, and the Cell Control (FA Test) flasks.

4.4.2 Rinse the FA Test flasks with PBS; decant the liquid.

4.4.3 Fill the FA Test flasks with 80% acetone and incubate at room temperature for 15 ± 5 minutes.

4.4.4 Decant the 80% acetone from the FA Test flasks and air dry at room temperature.

4.4.5 Pipette 2 mL of working PRV Conjugate into the FA Test Flasks and incubate for 45 ± 15 minutes at 36°± 2°C in a CO₂ incubator.

4.4.6 Decant and add 20 mL of PBS to the FA Test flasks and rotate back and forth to rinse the cells.

4.4.7 Repeat Step 6 for a total of 5 washes.

4.4.8 Rinse the FA Test flasks in DI, decant, and allow to air dry or dry at 36°± 2°C.

4.4.9 Examine the FA Test flasks with a UV microscope.

4.4.10 A flask is considered positive for PRV if typical, nuclear, apple-green fluorescence is observed in any cell.

4.4.11 For a valid FA test, the PRV Positive Control must show typical, apple-green fluorescent infected cells.

4.4.12 For a valid FA test, the HAT Medium flask and the Cell Control flask must not show fluorescence.

4.4.13 If either Section 4.4.11 or Section 4.4.12 criteria are not met, the test is considered a NO TEST and the entire test, starting with a new vial of the Test Vaccine, is repeated.
Interpretation of the Test Results

5.1 For a valid test, the HAT Medium flask and MDBK Cell Control must remain free of CPE and all flasks must remain free of bacterial or fungal contamination.

5.2 The PRV Positive Control must show CPE after passage in the MDBK cells.

5.3 For a SATISFACTORY test, a Test Vaccine must not show CPE after passage in MDBK cells.

5.4 Any Test Vaccine exhibiting typical PRV CPE in the initial test and found positive in a valid FA Confirmatory Test is retested in duplicate using 2 new vials of the Test Vaccine.

5.4.1 If either of the 2 retests of the Test Vaccine exhibits typical PRV CPE and is found positive in a valid FA Confirmatory Test, the Test Vaccine is UNSATISFACTORY.

5.4.2 If no typical PRV CPE is observed in either of the 2 retests, the Test Vaccine is SATISFACTORY.

5.5 Any Test Vaccine exhibiting typical PRV CPE in the initial test and found negative in a valid FA Confirmatory Test is considered INCONCLUSIVE for TK-activity. Repeat Section 4.3.1 and examine for possible viral contamination.

Report of Test Results

Record all test results on the test record.

References


8. Summary of Revisions

Version .05

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

Version .04

- The Contact information has been updated.

Version .03

- The Contact has been changed from Kenneth Eernisse to Joseph Hermann.
- 2.1: The pipettor has been removed from the list of equipment/instrumentation used for this test.

Version .02

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- 2.2.4.2 The amount of sodium bicarbonate (NaHCO$_3$) has been changed from 2.2 g to 1.1 g.
- 2.2.4.7 L-glutamine has been added. Penicillin and streptomycin have been removed.
- 2.2.5.2 L-glutamine has been removed.
- 2.2.11 “Syringe and needle” has been changed to “graduate cylinders”
- “Reference and Reagent Sheet” has been changed to “Reagent Data Sheet” throughout the document.
- “Test Serial” has been changed to “Test Vaccine” throughout the document.
- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.